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STACHYOSE

Stachys tuberifera
(Chinese artichoke)

(Lupeose; a-D-Gal-[1->6]-a-D-Gal-[1->6]-a-D-Glc-[1->2]-ß-D-Fru)

Prepared by a modification of the method of von Plante, A. and Schulze, E., Ber., 23, 1962.

AVAILABLITY

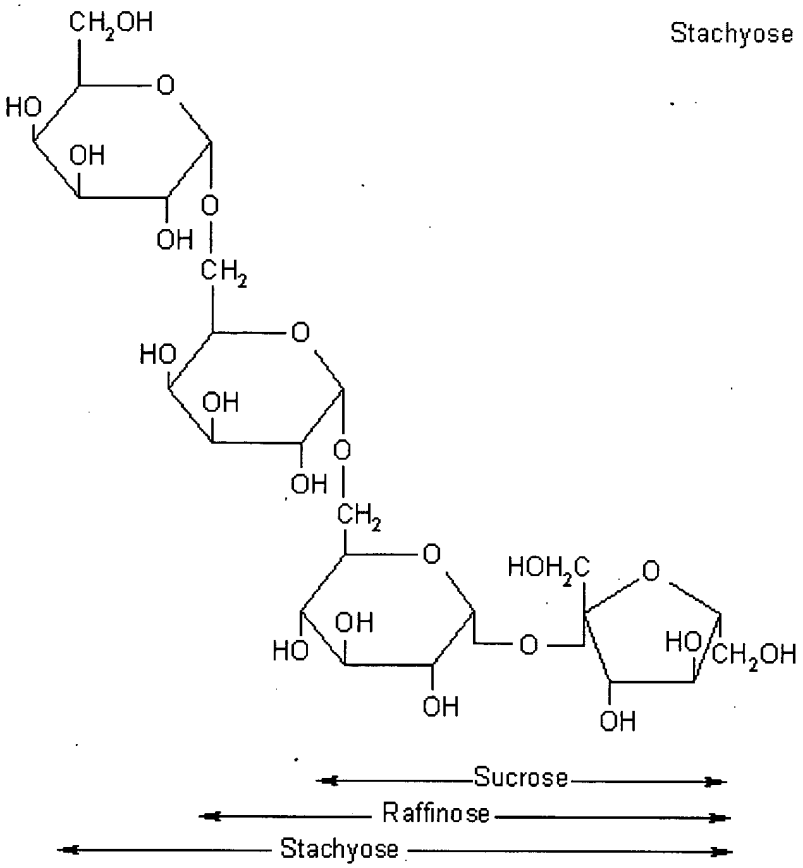
1. Source: Chinese Artichoke

Form: Crystalline, hydrate
Solubility: Readily soluble in distilled water or dilute buffer
Stability: 4°C
Catalog No.: 229J0000

CALZYME RETURN

Last Revised - 1/1/98

Stachyose



Stachyose, [*O*-α-D-galactopyranosyl-(1->6)-*O*-α-D-galactopyranosyl-(1->6)-α-D-glucopyranosyl-β-D-fructofuranoside], is a tetrasaccharide found coexists with raffinose and other related oligosaccharides in various organs of a number of plants. It is a major oligosaccharide in several plant species. Stachyose and other oligosaccharides of the raffinose family have been recognized as important transport carbohydrates in many woody plants, cucurbits, labiates, and legumes.

Stachyose synthesized in the leaves can be transported to other organs where it serves various functions. It can act as a storage carbohydrate in the storage organs such as roots and seeds. The accumulation of stachyose, along with sucrose and raffinsoe, in leaves can also provide frost-hardiness to winter-hardy plants.

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Oligomerization Process of the Hemolytic Lectin CEL-III Purified From a Sea Cucumber, *Cucumaria echinata*[1]

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CEL-III is a Ca^{2+}-dependent lectin purified from a sea cucumber, *Cucumaria echinata*. This protein exhibits strong hemolytic activity as well as cytotoxicity toward some cultured cell lines. Hemolysis is caused by CEL-III oligomers formed in the cell membrane after binding to specific carbohydrate chains on the cell surface. We have found that the oligomerization of CEL-III is also induced by the binding of simple carbohydrates, such as lactose, in aqueous solution under high pH and high ionic strength conditions. From gel filtration analysis of the oligomerization of CEL-III, it was found that the formation of the CEL-III oligomer is effectively induced by the binding of lactose and lactulose, disaccharides containing a β-galactoside structure. Electron micrographs of the resulting oligomers revealed them to exist as particles with a size of approximately 20-30 nm. The oligomerization process required more than 1 h, which is consistent with the increase in surface hydrophobicity as measured using a fluorescent probe, 8-anilinonaphthalene-1-sulfonate. However, a change in the far-UV CD spectra as well as small-angle X-ray scattering occurred within a few minutes, suggesting that a structural change in the protein takes place rapidly, but the following growth of the oligomer is a much slower process.

Key words: hemolysis, lectin, oligomer, sea cucumber, small-angle X-ray scattering.

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CEL-III is a Ca^{2+}-dependent lectin purified from a sea cucumber, *Cucumaria echinata*. This lectin shows hemolytic activity as well as cytotoxicity toward some cultured cell lines (1, 2). The sensitivity of cells to the cytotoxicity of CEL-III varies;

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some cell lines show considerable resistance to the toxicity of this protein. This may be due to differences in the receptor molecules expressed on target cell membranes (3). CEL-III binds to carbohydrate chains on the cell surface, leading to its oligomerization and concomitant insertion into the cell membrane. The amino acid sequence of CEL-III determined from both the protein and the cDNA indicates that the N-terminal two-thirds of CEL-III has homology to the sequences of the B-chains of ricin and abrin, plant lectins with high toxicity, while the C-terminal region is relatively hydrophobic, and is assumed to function as an oligomerization domain (4). The N-terminal domain has six tandem repeats of 40-50 amino acid residue units, and probably contains two carbohydrate-binding sites (5), as in the B-chains of ricin and abrin (6, 7). We have identified lactosyl ceramide as the most efficient receptor in human erythrocyte membranes in terms of the induction of hemolysis by CEL-III (8). Similarly, some glycolipids have been found to be specific receptors for bacterial toxins (9-12). Probably, it is more advantageous for toxins to bind to glycolipids rather than glycoproteins for interaction with cell membranes, which is essential for their toxic action. In the case of CEL-III, an oligomer with a molecular mass of about 270 kDa is detected in the membranes of cells treated with CEL-III (13). Such oligomers were hardly detected when CEL-III was treated with specific carbohydrates in aqueous solution under physiological conditions. Therefore, it is very likely that, under physiological conditions, interaction with a lipid bilayer is required for CEL-III oligomerization in addition to binding to the carbohydrate chains of receptor glycolipids. On the other hand, it was found that CEL-III oligomerization also occurs in aqueous solution in the absence of cell membranes, when the protein binds specific carbohydrates such as lactose and *N*-acetyllactosamine under high pH and high ionic strength conditions (13). This indicates that not only binding to specific carbohydrate chains on the cell surface, but also additional factors, such as a partially hydrophobic environment or high pH and high salt concentration, are necessary for conformational change and the resulting oligomerization of CEL-III. In this work, we investigated the carbohydrate-induced oligomerization of CEL-III in aqueous solution as a model system to obtain further insight into the interaction between CEL-III and cell membranes that underlies its hemolytic action.

MATERIALS AND METHODS

Purification of CEL-III

Specimens of *C. echinata* were collected in the Sea of Genkai (Fukuoka). CEL-III was purified from extracts of *C. echinata* homogenates by column chromatography on lactosyl-Cellulofine, GalNAc-Cellulofine, and Sephadex G-75, essentially as previously described (3). Chromatography was performed at 4°C. The purified protein was stored frozen in 10 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl (TBS) at -20°C. Protein concentration was calculated based on the absorbance value of 1.4 at 280 nm for a 0.1% (w/v) protein solution in TBS, which was determined with bicinchoninic acid by the method of Smith *et al.* (14) using bovine serum albumin as a standard.

Gel Filtration of CEL-III Oligomer

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CEL-III oligomer was prepared by incubating the protein with 10 mM lactose at 20°C for 30 min in 20 mM glycine-NaOH buffer, pH 10, containing 1 M NaCl, and 10 mM $CaCl_2$. The resulting oligomer was applied to a Superose 12HR 10/30 column (Pharmacia) equilibrated with TBS, and eluted with the same buffer at a flow rate of 0.5 ml/min.

Transmission Electron Microscopy

CEL-III oligomer prepared by incubating the protein with lactose as described above was dialyzed against TBS, then applied to a carbon-coated copper grid. Staining was done with 1% (w/v) uranyl acetate, and the grids were examined using a JEOL JEM-100S transmission electron microscope (100 kV).

Measurement of Surface Hydrophobicity with 8-Anilinonaphthalene-1-Sulfonate (ANS)

The surface hydrophobicity of CEL-III was measured with a fluorescent probe, ANS. A CEL-III solution (0.3 mg/ml) was mixed with ANS (40 μM) in 20 mM glycine-NaOH, pH 10, containing 1 M NaCl and 10 mM $CaCl_2$, and the fluorescence at 490 nm was recorded, with excitation at 380 nm at 25°C. Measurements were carried out in the presence or absence of 10 mM carbohydrates.

Circular Dichroism (CD) Spectroscopy

CD spectra of the CEL-III monomer and oligomer were recorded at 20°C using a JASCO J-720 spectropolarimeter. CEL-III in 20 mM glycine-NaOH, pH 10, containing 1 M NaCl and 10 mM $CaCl_2$ was mixed with 10 mM solutions of different carbohydrates, and the spectra were recorded after 1 min or 1 h.

Small Angle X-Ray Scattering

SAXS measurements were done at the RIKEN structural biology beamline I (BL45XU-SAXS)/SPring-8 (15). This beamline uses ideal SAXS optics very suitable for collecting X-ray scattering data in a very small-angle region. The X-ray wavelength was 1.0 Å. The camera length used was 2.2 m. All SAXS patterns were collected by a XR-II+CCD detector and were circular averaged to one-dimension, then scaled by incident X-ray intensity (16). Data collection time was 2 s. 130 μl of a CEL-III solution (0.9 mg/ml) in 20 mM glycine-NaOH buffer, pH 10, containing 1 M NaCl and 10 mM $CaCl_2$ was mixed with 10 μl carbohydrates. We checked the radiation damage to CEL-III with/without carbohydrates by the time dependencies of the SAXS images; they were stable within 10 s. The radius of gyration, *Rg*, which equals the second momentum of the electron density of CEL-III, was determined using the Guinier approximation,

$$I(S) = I(0)\exp(-4\pi^2 Rg^2 S^2 / 3)$$

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where, S is an angular momentum defined by $S = 2\sin\upsilon/\lambda$: 2υ is the scattering angle, λ is a wavelength (17). The forward scattering, $I(0)$, is proportional to molecular weight.

RESULTS

Time Course of the Oligomerization of CEL-III

We have previously found that CEL-III has carbohydrate-binding activity over a wide range of pH (pH 4-10) (5, 18). When CEL-III bound specific carbohydrates, such as lactose and *N*-acetyllactosamine, at pH 10 in the presence of 1 M NaCl, effective oligomerization of the protein was observed (13). In the present work, we examined the effects of several carbohydrates on the time-course of the formation of CEL-III oligomer by gel filtration. Figure 1, A and B, shows representative elution profiles of the proteins from a Superose 12HR column. After incubation for various times with 10 mM carbohydrates at pH 10 in the presence of 1 M NaCl, CEL-III was applied to the column. As seen in these chromatograms, the peak of CEL-III oligomer appeared at 14.5 min with a concomitant decrease in the size of the monomer peak at 24 min in the case of lactose and lactulose. The size of the oligomer was estimated to be over 300 kDa, by comparison with the standard proteins (data not shown). The amount of oligomer increased with time and nearly reached a plateau after 1 h. Oligomerization was also evident with phenyl β-galactoside in addition to lactose and lactulose (Fig. 1C). The most efficient oligomerization was induced by lactulose, followed by lactose among the carbohydrates tested, while *N*-acetylgalactosamine and galactose induced only small amounts of the oligomer. Although melibiose can bind to CEL-III with moderately high affinity (3, 18), almost no oligomer peak was observed. This is in agreement with the results of SDS-PAGE of carbohydrate-treated CEL-III (13), confirming the importance of the configuration of the galactosidic linkage for induction of oligomerization.

Electron Microscopy of CEL-III Oligomers Induced by the Binding of Lactose

The CEL-III oligomer formed upon the binding of lactose was observed by electron microscopy after negative staining with uranyl acetate. As shown in Fig. 2A, CEL-III oligomers appeared as particles with dimensions of about 20-30 nm. This is comparable to the size estimated by SAXS analysis (19), in which the oligomer formed by the binding of lactose gave a maximum dimension of 29 nm. Although the spatial arrangement of the protein molecules in the oligomers was unclear, several negatively stained regions were found on the surface of the oligomers. It may be possible that such regions reflect the hollow structure of the oligomers that is responsible for the hemolytic action of CEL-III. There was no apparent difference in the shape of the oligomers induced by the binding of lactose and lactulose (data not shown). Such oligomers were not observed when CEL-III was treated in the same manner without carbohydrate (Fig. 2B).

Changes in the Surface Hydrophobicity of CEL-III Due to Oligomerization

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Figure 3 shows the change in the surface hydrophobicity of CEL-III oligomers as measured using a fluorescent probe, ANS. ANS was added to CEL-III solutions at appropriate intervals after mixing with lactose or lactulose, and the change in the fluorescence at 490 nm of the ANS was measured. As a result, a time-dependent increase in surface hydrophobicity was observed. The profile of the increase was very similar to that of the increase in the oligomer as analyzed by gel filtration (Fig. 1C). This confirms that the oligomerization of CEL-III accompanies surface hydrophobicity, as reflected in the increase in ANS fluorescence.

CD Spectral Changes of CEL-III

Since it has been suggested that the CEL-III oligomer has an increased β-sheet structure compared with the monomer (13), the influence of several carbohydrates on the CD spectrum of CEL-III was examined. Figure 4 shows the far-UV CD spectra of CEL-III before and after the binding of several carbohydrates at pH 10 in the presence of 1 M NaCl. Upon the binding of lactose and lactulose, the molar elipticity around 207 and 227 nm increased, suggesting an increase in β-sheet structure (20). In contrast, glucose and melibiose did not cause such a change, although the former has a moderate affinity for CEL-III as mentioned above. The change in the CD spectrum occurred within 1 min after the addition of carbohydrate, and no further significant changes were observed after 1 h. This is in contrast to the increase in the oligomer as detected by gel filtration analysis, as well as the increase in surface hydrophobicity, both which continued for more than 1 h, suggesting that the change in the secondary structure of CEL-III may be involved only in the initiation step of oligomerization.

Structural Changes Monitored by SAXS

The rapid conformational change associated with the oligomerization of CEL-III was also examined by synchrotron SAXS. As shown in Fig. 5, when the SAXS of CEL-III was measured at pH 10 in the presence of 1 M NaCl, marked changes in the scattering curves in the range of 0.002-0.005 $Å^{-1}$ were found immediately after the addition of lactose and galactose (*ca.* 1 min), while glucose and melibiose induced little change. Table I summarizes the apparent *Rg* values after mixing with carbohydrates. In the case of lactose, an *Rg* of 102.91 Å was the same as the value reported previously (19). The *I*(0) value was larger than that of the monomer (22.67), which was also same as that of the equilibrated value (19). This indicate that CEL-III oligomers had already accumulated within 1 min of lactose binding. For galactose, the *Rg* of 92.46 Å is similar to that with lactose, while the *I*(0) value is much smaller. The oligomer formed with galactose is similar to that with lactose, but the CEL-III does not oligomerize completely. No accumulation of oligomer was observed with glucose. Melibiose induced the formation of oligomer, though the size seems smaller than those induced by lactose and galactose. These results indicate that the oligomerization of CEL-III may begin within a very short time after the addition of carbohydrates.

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DISCUSSION

Upon binding specific carbohydrates, such as lactose, under high pH and high ionic strength conditions, CEL-III forms oligomers of uniform size in aqueous solution. The size of the oligomers is similar (about 270 kDa) on SDS-PAGE as that detected in erythrocyte membranes treated with CEL-III, suggesting that both types of oligomers result from the same mechanism. Therefore, carbohydrate-induced oligomerization can be expected to become a good model system to investigate the oligomerization mechanism of CEL-III in cell membranes. Our previous SAXS study (19) revealed that CEL-III oligomers formed in aqueous solution consist of about 21 CEL-III molecules (approximately 1 MDa), which is much larger than that estimated on SDS-PAGE (13). This leads to the speculation that CEL-III oligomers in aqueous solution could consist of 3 or 4 subunits, each of which is composed of six CEL-III molecules (285 kDa) held by strong interactions. As monitored by gel filtration analysis (Fig. 1), the formation of CEL-III oligomers appears to be a relatively slow process, requiring more than 1 h to reach a maximum. However, changes in the CD spectrum of CEL-III induced by the binding of lactose occur within a few minutes. This difference suggests that the conformational change in CEL-III takes place rapidly, but the following accumulation of the oligomer is much slower. Since SAXS indicated the formation of oligomers with Rg values similar to that reported previously (19) after mixing with lactose, an oligomer of high molecular mass should be formed at least partially at the beginning of this process. As previously reported (13), treating erythrocytes with CEL-III for a long time causes severe damage to the cell membranes. This supports the idea that the hemolytic action of CEL-III is not due only to colloid-osmotic rupture of the membrane caused by small membrane pores, but also by the destruction of the membrane structure by large oligomers that slowly accumulate through lateral diffusion in the membrane.

The formation of CEL-III oligomers is effectively promoted by the binding of carbohydrates that contain terminal β-1,4 galactosidic linkages, such as lactose and lactulose, whereas melibiose, which has an α-1,6 linkage, fails to induce the oligomerization of CEL-III. This observation implies that the configuration about C1 of the nonreducing ends of the carbohydrate chains is very important in triggering the oligomerization of CEL-III. The importance of a glycosidic linkage at the nonreducing ends of the carbohydrate chains is also apparent from the observation that lactosylceramide is the most efficient receptor of CEL-III for pore-formation in liposomes containing human erythrocyte lipids, whereas a glycolipid containing an α-1,3 linkage (Gb_3Cer) exhibits much poorer ability (8). Monosaccharides, such as galactose and *N*-acetylgalactosamine, are not effective inducers of oligomerization, although *N*-acetylgalactosamine exhibits the highest affinity for CEL-III in hemagglutination inhibition assay (3) as well as carbohydrate-coated microplate binding assay (18). It is interesting that oligomerization is effectively induced by phenyl β-galactoside, indicating that the stacking interaction of a phenyl group with the binding site of the protein plays an important role in the conformational change necessary for oligomerization. Probably, such a rather nonspecific hydrophobic interaction is essential at the binding subsite located next to the β-galactoside-

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recognition site of CEL-III, which accommodates the glucose and fructose moieties of lactose and lactulose, respectively. This may be why different carbohydrate moieties at the reducing ends have similar abilities to induce oligomerization.

Electron microscopy revealed that the CEL-III oligomer has dimensions of 20-30 nm with some hollow regions on the surface (Fig. 2A). This observation is consistent with the results of the SAXS study (19), in which the maximum dimension of CEL-III was estimated to be 29 nm. Furthermore, the presence of some pore-like regions observed in the electron micrograph also seems to be related to the fact that the theoretical scattering curve derived from a hollow structure gave the best fit to the observed scattering data. Although the actual structure of the membrane pores formed by CEL-III may be somewhat different, it seems likely that the CEL-III oligomer in solution induced by the binding of carbohydrates has a close relationship with the pore-forming oligomer in the cell membrane.

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